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13. ABSTRACT (Maximum 200 words) The following were the research activities conducted during the first year of the Cooperative Agreement DAMD17-93-8001: <u>Project I</u> Cohorts of young soldiers serving in Israel Defense Force field units and naturally exposed to enteric pathogens have been maintained under intensive clinical and laboratory surveillance during the summer months of years 1997 and 1998. During this period, the incidence of shigellosis ranged between 24 and 67 cases per 1,000 per 3-5 summer months while the incidence of gastroenteritis associated with ETEC ranged between 57 and 63 per 1,000. <u>Laboratory core</u> <u>Study 1:</u> A collection of 392 <i>Shigella</i> and 137 ETEC strains isolated from subjects under surveillance during 1993-1997 were tested for antibiotic susceptibility. <u>Study 2:</u> Nalidixic acid (NA) resistant <i>S. sonnei</i> strains isolated during an outbreak occurring in a pediatric population, were examined and compared to NA sensitive <i>S. sonnei</i> strains isolated in the same region. The molecular basis of the antimicrobial resistance to nalidixic acid is currently investigated in our lab. <u>Study 3:</u> A PCR protocol for direct and rapid detection of shiga-like toxin genes in stool specimens was developed and used to identify SLT II in stools of children involved in an outbreak of diarrhea associated with <i>E. coli</i> O157 and involving a case of hemolytic uremic syndrome. <u>Study 4:</u> <i>Shigella</i> LPS antibodies, elicited by immunization with an experimental vaccine composed of <i>S. sonnei</i> or <i>S. flexneri</i> 2a O-specific polysaccharide conjugated to <i>Pseudomonas aeruginosa</i> recombinant exoprotein, were quantified and characterized. <u>Study 5:</u> The antibody-mediated killing of <i>Shigella</i> by phagocytes was tested using serum preparations containing different ratios of <i>Shigella</i> IgG1 and IgG2 antibodies. It was found that relatively high levels of IgG1 are crucial for efficient killing of the homologous <i>Shigella</i> organisms. <u>Study 6:</u> Consecutive serum and urine samples of subjects involved in a phase II study of the live attenuated <i>S. flexneri</i> 2a SC602 vaccine that was carried out at WRAIR and USAMRIID were tested by ELISA for specific <i>S. flexneri</i> 2a antibodies at the Army Health Branch Research Unit.					
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## **Project 1**

### **Intensive surveillance of target populations for field evaluation of vaccines against shigellosis and enterotoxigenic *E.coli*-associated diarrhea**

#### **Statement of Work**

##### **Goal:**

To provide surveillance data necessary for the design of vaccine efficacy trials against shigellosis, enterotoxigenic *E. coli*-associated diarrhea and other enteric diseases.

##### **Objectives:**

1. To maintain the present logistic infrastructure including sample collection ability at the field sites, transport facilities to the research lab, samples and data processing at the research lab
2. To determine yearly incidence rates of shigellosis, ETEC-associated diarrhea and other diarrheal diseases in the candidate populations for vaccine trials
3. To determine the relative importance of the *Shigella* serogroups and serotypes in the etiology of shigellosis
4. To determine the relative importance of the LT and ST producing ETEC, the prevalent ETEC serotypes and colonization factor antigens involved in ETEC infections.
5. To identify the reservoir and modes of transmission of *Shigella* and ETEC in the field using molecular methods such as ribotyping.
6. To compare the clinical picture of disease caused by various enteropathogens
7. To monitor the patterns of antimicrobial susceptibility of *Shigella*, enterotoxigenic and enterohemorrhagic *E.coli*, *Campylobacter* and *Salmonella* isolates.
8. To identify new field sites for evaluation of vaccine efficacy

## Introduction

Diarrheal diseases are an important medical problem for all military personnel on duty in the developing world or in areas where sanitation is inadequate. Two of the major components of diarrheal diseases occurring in the military are shigellosis (dysentery and diarrhea caused by *Shigella*, characterized by severe, occasionally bloody, diarrhea accompanied by fever) and traveler's diarrhea (diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC), characterized by watery diarrhea occurring in people changing their living conditions for the worse). As yet, there are no licensed vaccines against either of these enteric infections.

American troops experienced high attack rates of diarrheal diseases during different military operations in the Middle East [1,2,3]. Diarrhea rates of over 50% were experienced in many of the units under surveillance making diarrhea the most important medical problem encountered during ODSS. Bacteriological studies showed that a bacterial pathogen was isolated in 50% of troops with diarrhea. Enterotoxigenic *Escherichia coli* was isolated in 21%, *Shigella* species in 19% and dual infections in 9%. *Salmonella* and *Campylobacter jejuni* were isolated from less than 2% of troops [1].

Of 132 cases of ETEC diarrhea, 45% were diagnosed as ETEC which produce heat-labile toxin (LT) and heat-stable (ST) toxin, 40% produced ST only, and 15% produced LT only [2]. Known colonization factor antigens (CFA) were detected in 68% of ETEC isolates. CFA I was detected in 9% of isolates, CFA II (34%), and CFA IV (33%).

The military importance of shigellosis is well documented. Despite a decline over the past few decades in the severity of its most serious clinical manifestation, bacillary dysentery and shigellosis continue to pose a major threat to the operational capability of military units [1,4,5,6,7]. Although the mortality rate of shigellosis has declined significantly, the substantial morbidity encountered under field conditions in endemic areas such as the Middle East, continues to pose serious operational problems. Further, the rapid emergence of multiple-antibiotic-resistant strains has made treatment more difficult and involving expensive antibiotics with less desirable safety characteristics.



Adequate conditions of sanitation and hygiene should be sufficient to prevent shigellosis and traveler's diarrhea. However, in military populations serving in field units it is frequently difficult, if not impossible, to provide such conditions. In such circumstances, effective vaccination is the only reliable means of preventing outbreaks.

### **The situation in Israel**

In addition to the problem encountered by the military under field conditions, Israel is a highly endemic area for shigellosis. In 1980 47.4 per million American residents were isolated with *Shigella* [8]. In Israel, ~1000 cases of shigellosis per million residents are reported annually [9]; i.e., ~20 times higher than in the USA. Soldiers serving under field conditions [10] and children aged 1-4 [11] are at increased risk of developing shigellosis. Shigellosis tends to be more severe with occasional mortality among young children at nursery school age. An effective vaccine will be useful for preventing shigellosis in infants and young children, as well as in adults serving in the military. The follow-up studies carried out during the last years among Israeli soldiers serving under field conditions revealed a high attack rate of diarrheal diseases. Approximately 90% of the subjects maintained under surveillance for periods of 10 weeks, recalled at least one episode of diarrhea (3 or more loose stools per 24 hours) during the follow-up. These episodes have the features of traveler's diarrhea. They occur among subjects who change radically their living conditions moving from a regular civilian environment or military units with good sanitation to difficult field conditions. In addition to shigellosis, non-*Shigella* diarrhea accounts for an important part of the enteric diseases. The aim of the follow-up studies on diarrheal diseases occurring among Israeli soldiers serving under field conditions was to provide ongoing data surveillance necessary for the design of vaccine efficacy trials against shigellosis, enterotoxigenic *E.coli*-associated diarrhea and other enteric diseases.

### **Methods**

**Study population:** Cohorts of soldiers, primarily new recruits, serving in 4-5 field units who were kept under clinical and laboratory surveillance for periods of 3-7 months during their summer training cycles in the field. Those presenting at the unit clinic with diarrhea were examined and stool cultures obtained. In addition, information on recalled episodes of diarrhea during the training cycles was obtained by a questionnaire

filled by the soldiers at the end of the follow-up period. Epidemiological studies carried out in the last years have demonstrated high incidences of shigellosis and ETEC-associated diarrhea among soldiers at these sites.

**Sample size:** Two cohorts of about 500-800 subjects each were followed-up each summer for a period of 3-7 months.

**Laboratory procedures:** Fecal swabs were processed on receipt and incubated overnight at 35-37°C on MacConkey, XLD and *Campylobacter* agars. *Shigella*, *Salmonella* and *Campylobacter* were identified by standard methods. The isolates were confirmed by the Reference Laboratories of the Israel Ministry of Health. PCR protocols developed at the Army Health Branch Research Unit were used for direct detection of ETEC in the stool specimens.

## Results

During the surveillance period of 1997, 717 (48%) of the 1,500 subjects maintained under surveillance complained of at least one episode of gastrointestinal symptoms (total of 860 episodes of either diarrhea, nausea, vomiting or stomachache) (Table 1). Stool samples were obtained from 683 (79.5%) of these patients. One hundred (15%) of the stool specimens were positive for *Shigella* spp. and 140 (20.5%) for ETEC. Of the 100 isolates of *Shigella* spp. 13 were defined as *S. sonnei*, 70 as *S. flexneri*, 5 as *S. boydii* and 11 as *S. dysenteriae*. One stool specimen harbored both *S. sonnei* and *S. flexneri* organisms (Table 1). The relative frequency of detection of genes encoding for ST and LT in the 140 stool samples positive for ETEC by PCR was the following: 84 (60%) were LT-ST+, 30 (22%) were LT+ST- and 26 (18%) were LT+ST+. Fifteen specimens positive for ETEC were also culture-positive for *Shigella* spp. The incidence rates of shigellosis and ETEC-associated gastroenteritis for the surveillance period of summer 1997 were 67 per 1,000 and 93 per 1,000, respectively.

During the surveillance period of 1998, 317 (21%) of the 1,486 subjects maintained under surveillance complained of gastrointestinal symptoms (total of 329 episodes of either diarrhea, nausea, vomiting or stomachache) (Table 1). Stool samples were obtained from 303 (91.5%) of these patients. Thirty-six (12%) of the stool specimens were positive for *Shigella* spp. and 57 (19%) for ETEC (Table 1). Of the 36 isolates of *Shigella* spp. 17 were defined as

*S. sonnei* and 19 as *S. boydii*. The relative frequency of detection of genes encoding for ST and LT in the 57 stool samples positive for ETEC by PCR was the following: 27 (47%) were LT-ST+, 20 (35%) were LT+ST- and 10 (18%) were LT+ST+. three specimens positive for ETEC were also culture-positive for *Shigella* spp. and another one was positive for *Campylobacter* spp.. The incidence rates of shigellosis and ETEC-associated gastroenteritis for the surveillance period of summer 1998 were 24 per 1,000 and 57 per 1,000, respectively.

**Table 1:** Detection rate of various enteropathogens occurring in soldiers serving in field units.

	1997 (N=1500)	1998 (N=1486)
Cases of gastroenteritis	860	329
Stools Collected	683 (79.5%)	303 (91.5%)
ETEC*	140 (20.5%)	57 (19%)
<i>Shigella</i> spp.	100 (15%)	36 (12%)
<i>Campylobacter</i> spp.	0 (0%)	2 (0.7%)
<i>Giardia lamblia</i>	10 (1.5%)	5 (1.7%)
<i>Entamoeba</i> spp.	1 (0.15%)	0 (0%)

\*Including 15 stools that were culture-positive for *Shigella* (1997), 3 stools that were culture-positive for *Shigella* (1998), and a single stool that was positive for *Campylobacter* (1998).

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## Laboratory Core

### Statement of Work

#### Goal

To provide and maintain a high level of laboratory expertise needed to support the different projects of the Research Unit

#### Objectives

1. Performance of the microbiological, molecular and immunological tests on stool, urine, whole blood and serum samples obtained from volunteers under intensive surveillance (project 1).
2. Development and field evaluation of rapid diagnostic tests using advanced technology. Development, refinement and field evaluation of PCR protocols for detection of *Shigella* various serogroups, enterotoxigenic *E.coli*, enterohemorrhagic *E. coli*, *Salmonella* spp., *Cryptosporidium parvum*, Norwalk virus.
3. Development and refinement of immunological correlates of protection against shigellosis and ETEC related diarrhea.
4. Transfer of new diagnostic technology to the Central IDF Lab or support in performance of the molecular and serological tests included in project 2.
5. Laboratory support to phase 2 and 3 studies of *Shigella* or ETEC vaccines carried out in Israel (project 3).
6. Laboratory support to *Shigella* vaccine studies carried out by the US Army.

## Study 1.

### Antimicrobial susceptibility patterns of *Shigella* and enterotoxigenic *E. coli* isolated from soldiers suffering from diarrhea

#### Background

The antimicrobial susceptibility patterns of a collection of 392 *Shigella* and 137 ETEC strains isolated from stool samples of subjects maintained under surveillance for diarrheal diseases during the summers of 1993-7 were examined.

#### Method

**Determination of antibiotic susceptibility:** Susceptibility of the *Shigella* and ETEC isolates to the various antimicrobial drugs was performed by the Kirby & Bauer disk diffusion method (1) following the NCCLS recommendations. The following drugs were investigated: ciprofloxacin (cip), norfloxacin (Nor), ampicillin (Amp), trimethoprim-sulphamethoxazole (Tmp/Smx), tetracycline (Tet), nalidixic acid (Nal), amoxicillin-clavunolate (Amo/clav) and chloramphenicol (Chl)

#### Results

Sixty-six percent, 16% and 94% of the *S. sonnei* and 93%, 77% and 69% of the *S. flexneri* isolates were resistant to tetracycline, ampicillin and trimethoprim-sulphamethoxazole, respectively. About half of the other *Shigella* serogroups were resistant to tetracycline, ampicillin and trimethoprim-sulphamethoxazole. None of the *S. sonnei*, *S. flexneri* and other *Shigella* isolates was resistant to quinolones and fluoroquinolones such as nalidixic acid, norfloxacin and ciprofloxacin (table 1). Seventy-five percent of the *Shigella* isolates were resistant to more than one antibiotic (table 2). Thirty three percent were resistant to three antibiotics (tetracycline, ampicillin and trimethoprim-sulphamethoxazole) and 42% were resistant to two antibiotics (31% to tetracycline and trimethoprim-sulphamethoxazole, and 5% to tetracycline and ampicillin and 6% to ampicillin and trimethoprim-sulphamethoxazole).

**Table 1:** Antibiotic resistance of *Shigella* isolates in the years 1993-7

	Tet	Nal. acid	Amp.	Tmp/Smx	Nor.	Cip.
<i>S. sonnei</i>	125/190 (66%)	0/189 (0%)	31/188 (16%)	175/187 (94%)	0/179 (0%)	0/158 (0%)
<i>S. flexneri</i>	152/163 (93%)	0/162 (0%)	125/163 (77%)	112/163 (69%)	0/148 (0%)	0/143 (0%)
Others	21/33 (64%)	0/32 (0%)	20/33 (61%)	21/33 (64%)	0/21 (0%)	0/31 (0%)

**Table 2:** Patterns of antibiotic resistance of *Shigella* isolates in the years 1993-7

	Antimicrobial resistance pattern			
Shigella serogroups	Amp + Sxt N=21	Amp+ Te N=20	Te+ Sxt N=119	Te + Amp+Sxt N=134
<i>Shigella sonnei</i> n=195	20 (10%)	1 (1%)	113 (58%)	10 (5%)
<i>Shigella flexneri</i> n=164	0	18 (11%)	4 (2%)	106 (65%)
<i>Shigella boydii</i> n=10	1 (10%)	0	2 (20%)	5 (50%)
<i>Shigella dysenteriae</i> n=23	0	1 (4%)	0	13 (57%)



The rates of ETEC strains resistant to the various antimicrobial drugs is presented in table 3. The highest rates of resistance were found against ampicillin, trimethoprim-sulfamethoxazole and tetracyclin. Two strains were resistant to nalidixic acid but none to ciprofloxacin. Fifty four (39%) of the ETEC isolates were resistant to at least one antimicrobial drug. Thirteen (9%) were resistant to three or more antibiotics

**Table 3:** Antibiotic resistance of ETEC representative isolates

	<b>Tet</b>	<b>Nal. acid</b>	<b>Amp.</b>	<b>Tmp/Smx</b>	<b>Amo/clav</b>	<b>Chl</b>	<b>Cip.</b>
<b>ETEC</b>	24/137	2/137	40/137	27/137	5/137	9/137	0/137
	(18%)	(1%)	(29%)	(20%)	(4%)	(7%)	(0%)

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## Study 2

**Quantitative analysis of IgG class and subclass and IgA serum response to *Shigella sonnei* and *Shigella flexneri* 2a polysaccharides following vaccination with *Shigella* conjugate vaccines.**

### Background

Organisms belonging to genus *Shigella* are important agents of worldwide diarrheal epidemics, causing about 200 million cases of dysentery (fever, mucous, bloody stools or/and cramps) and approximately 650,000 deaths annually (1). The populations at high risk include infants and children in developing countries, refugees, and military populations under field conditions (2, 3, and 4).

Although the mechanism of immunity against shigellosis is still not clear, there is evidence that natural and experimental infections with *Shigella* confer type-specific immunity of limited duration (5, 2, 6), which indicates that the O-specific polysaccharide is a protective antigen. It is assumed that an efficacious *Shigella* vaccine should induce an immune response similar in nature, and at least of the same extent, as that induced by the natural infection. Previous studies have found that serum IgG, IgA and IgM antibodies are elicited by natural *Shigella* infection (7), and that significant serum levels of pre-existing antibodies from the non-IgM fraction that recognize *Shigella* somatic antigen, correlate with a diminished attack rate of the disease (8, 9). Anti-O specific polysaccharide antibodies are not distributed randomly among the IgG subclasses. Analysis of the IgG subclass distribution of young adults after natural exposure to *Shigella sonnei* or *Shigella flexneri* 2a revealed a different pattern of response. The dominant component of the anti-*S. flexneri* response was IgG2, whereas against *S. sonnei* the human host produced IgG1 accompanied by smaller amounts of IgG2 (9).

As yet there are no licensed vaccines against shigellosis and the various current approaches in development of *Shigella* vaccines reflect the different concepts on the protection against the disease (10). These include - Hybrid *Shigella-E. coli* (or *S. typhi*) strains (11, 12), *Shigella* strains with attenuating mutations (13, 14) and acellular vaccines based on lipopolysaccharide /polysaccharide (LPS/PS) antigens (15, 3). *Shigella* polysaccharide-protein conjugates are vaccine candidates consisting of proteins that elicit enhanced antibody responses against the poorly immunogenic

polysaccharide (10, 3). This type of parenteral vaccine has been recently evaluated by the Israel Defense Force (I.D.F) for safety, immunogenicity (16) and efficacy (17). The results showed that *Shigella* polysaccharides conjugated to genetically detoxified recombinant *Pseudomonas aeruginosa* exoprotein A (*rEPA*) vaccines are safe and immunogenic, and that the *S.sonnei-rEPA* protective efficacy was 74% (8). The objectives of the present study were to quantify and characterize the serum antibody response of volunteers following vaccination with *Shigella* conjugates. We studied the presence and kinetics of anti-LPS IgG class and subclass concentrations induced by immunization with *S. sonnei* or *S. flexneri* 2a O-specific polysaccharide-*rEPA* vaccines in a group of subjects with a low risk of concomitant natural exposure to *Shigella*.

### Materials and methods

**Study population:** Male volunteers with a low risk of natural exposure to *Shigella* between the ages of 18 and 22 years were chosen to participate in the phase 2 study of the *S. sonnei* and *S. flexneri* 2a conjugates (FDA Prot. 90CH173; BB-IND-3488) (16). The study was approved by the Committee for Research on Human Subjects of the Israel Defence Force Medical Corps, the Surgeon General's Human Subjects Research Review Board of the Department of the U.S. Army, the U.S. National Institutes of Health, and Food and Drug Administration.

The volunteers were randomly assigned to receive one intra-muscular injection of *S. flexneri* 2a-*rEPA* (lot-83190, n=49) or *S.sonnei-rEPA* (lot-51591, n=48). In a third group of volunteers the Engerix hepatitis B vaccine, licensed in Israel, was used as a control vaccine (n=62). Sera were obtained from the volunteers before and at 2 and 6 weeks, and 6, 12 and 24 months after vaccination, and stored at -30°C, until tested.

**Investigational vaccines:** Conjugates consisting of the O-specific polysaccharide of *S. sonnei* and of *S. flexneri* 2a bound to the recombinant exoprotein A of *P. aeruginosa* (*rEPA*) were constructed at the laboratory of Developmental and Molecular Immunity, National Institute of Child Health and Human Development, as described (3). Each 0.5-mL dose contained 25 µg of polysaccharide and 75 µg of protein dissolved in saline 0.01% thimerosal.

**LPS preparation:** LPS of *Shigella sonnei* (form 1) and *Shigella flexneri* 2a, isolated from stools during acute infections, was extracted by the hot phenol-water method described by Westphal and Jann (18).

**1. Purification of anti-*Shigella* LPS antibodies to be used as standards:** The procedure has been previously described by Robin et al (9), and included several steps.

A. The coupling of *Shigella flexneri* 2a or *Shigella sonnei* LPS to epoxy-activated sepharose 6B (Pharmacia Fine Chemicals, Inc. Piscataway N.J). Lipopolysaccharides from *Shigella flexneri* 2a and *Shigella sonnei* were coupled to the epoxy-activated sepharose 6b at a ratio of 30 mg ligand per 8 mL gel and reacted in a stoppered vessel using a water bath shaker (Tuttnauer Inc. Israel) for 16 hours at 40°C. The coupled gel was washed according to the Pharmacia protocol, and the active groups were blocked during an overnight incubation at 40°C-50°C with 1M ethanol amine (pH 8.0). Excess blocking agent was removed by washing the gel in at least three cycles of alternating pH consisting of a wash with 0.1M Acetate buffer (pH 4.0) containing 0.5M NaCl, followed by 0.1M Tris-HCl buffer (pH 8.0) containing 0.5M NaCl. The gel was suspended in saline and stored at 4°C.

B. Affinity purification of specific antibodies: Sera of volunteers after natural and laboratory diagnostic *S. sonnei* and *S. flexneri* 2a infection were used for purification of anti-*Shigella* lipopolysaccharide antibodies and passed over a 15-20 mL affinity column. The columns were washed with 100-120 mL of 0.15M NaCl, 50mM Tris HCl (pH 7.5), followed by 50 mL of 250mM NaCl and 50mM Acetate (pH 5.0), until the O.D at 280nm of the flow-through was less than 0.05. Lipopolysaccharide-specific antibodies were then eluted from the column with 40-60 mL of 3.5M MgCl<sub>2</sub>. Eluted antibodies were immediately dialyzed against PBS and concentrated, as needed, using Omegacell 30K (Filtron Technology Corporation, Northborough, MA 01532). Bradford test (19) was used to measure the concentration of protein in serum, washes and eluted fractions throughout all stages of the affinity chromatography.

C. Analysis of output:

1. Analysis by gel electrophoresis: Eluted samples were collected into pools and analyzed by gel electrophoresis to provide information about the content and purity of proteins in the specimen. The electrophoresis was performed using 10%

SDS-Polyacrylamide mini gel and each test included purified human IgG and IgA (Sigma) as controls, and a prestained M.W-SDS blue marker (Sigma). Proteins were then stained by the silver staining modified method of Wary et al (20).

2. Specificity of samples: Samples were tested for their specificity by ELISA (16) with some modifications (described below), using different heterologous *Shigella* LPS as antigens or by ELISA using purified

O-specific polysaccharide (O-SPs) preparations as antigens: *Shigella* LPS (10 mg/mL) was treated with DNase (Sigma-D4527), RNase (Merck-24686) and then with pronase (Sigma-P0390), followed by acid hydrolysis and centrifugation as described (21).

D. Quantitation of specific anti-*Shigella* LPS IgA, IgG and IgG subclass antibodies was performed using the radial immunodiffusion procedure with kits from Binding Site (Human IgG LL NanoRID Kit GT 004.3, Human IgG subclass LL NanoRID 008 and Human IgA LL NanoRID Kit GT 010.3, Birmingham, United Kingdom).

## **2.Measurement of *Shigella* LPS immunoglobulins in volunteers serum samples:**

Tests were carried out in two fold dilutions using ELISA, performed in polystyrene microtiter plates (Costar, model 3590, Cambridge, MA, USA), according to the method of Cohen et al (22) with some minor modifications. Briefly, 100µl of coating buffer (0.05M carbonate buffer, pH 9.6) containing 10 µg/mL of *S.sonnei* or *S.flexneri* 2a LPS was added to 96 wells, and the wells were incubated for 1hr at 37°C. After removal of the coating solution, the plates were incubated for 1hr at 37°C with 0.05M phosphate-buffer saline (PBS) supplemented with casein and bovine serum albumin (both at 5 g/liter) to block the remaining unbound plastic sites. The wells were then washed twice in phosphate-buffered saline-tween 20 solution. Inactivated sera were added to the wells in PBS, at a 1:50 dilution, and the wells were incubated overnight at room temperature. After four further washings, goat anti-human immunoglobulin G, A, or M conjugated to alkaline phosphatase (Kirkegaard & Perry Laboratories, Gaithersburg, Md.), or monoclonal mouse anti-human immunoglobulin G subclasses (IgG1, IgG2, IgG3, IgG4) conjugated to alkaline phosphatase (Zymed, CA, USA), at a 1:500 dilution, were added to the macrotiter wells. The plates were incubated overnight at room temperature, washed and ELISA was completed sequentially by the

addition of the enzyme-substrate solution containing *para*-nitrophenylphosphate (1 mg/mL) in diethanolamine buffer at pH 9.8 and 3M NaOH. Optical density was read at 405nm with an automatic ELISA bio-kinetics EL340 reader (Bio-Tek Instruments, USA). Serum samples taken from each subject before and after vaccination or natural exposure to bacteria were tested within the same assay, positive and negative control sera being included in every microtitration plate in each of the assays.

A calculated ELISA OD based on a linear regression analysis of eight two fold dilutions was determined and, in order to convert ELISA results to micrograms per milliliter of specific antibody, it was compared to similarly calculated values of standards with known immunoglobulin concentrations present on the same plate.

**Statistical analysis:** Statistical significance of differences in the geometric mean concentration of the IgG subclasses at various times was examined using Duncan's multiple range test.

## Results

### Quantitation of IgG, IgG subclass and IgA serum response to *Shigella* LPS :

Affinity purified human *S.flexneri* 2a and *S.sonnei* antibodies were used to quantitate the IgG class and subclass and IgA serum response after vaccination with *S.sonnei*-rEPA or *S.flexneri* 2a-rEPA, and to compare this immune response with that elicited by natural infection caused by the homologous organisms (9). It was found that administration of the *S. sonnei* and *S. flexneri* conjugates induced a 40 fold (3.8 to 115.8 µg/mL, n=35) and 11.4 fold (11.26 to 126.5 µg/mL, n=44) increase, respectively, in the geometric mean concentration of IgG antibodies to homologous LPS, 14 days after vaccination.

These antibodies persisted over a period of more than two years and their concentration was 7.5 and 3.1 fold higher as compared to the prevaccination titers and to day 720, respectively (figure 1 and figure 2). Analysis of serum IgA after *S. sonnei*-rEPA vaccination showed an increase from 0.92 µg/mL to a peak of 53.4 µg/mL at day 14(n=35) followed by a gradual decline to 12.8 µg/mL after two years. In the case of vaccination with *S.flexneri* 2a-rEPA an increase from 1.77 µg/mL to a peak of 36.6 µg/mL at day 14(n=44) was observed. The level decreased to 7.26 µg/mL after two years. The two conjugates induced a similar long-term response of

specific antibodies. No changes in serum IgG or IgA anti *S. sonnei* and anti *S. flexneri* 2a LPS levels were detected in recipients of the hepatitis (control) vaccine during the 2-year of follow-up.

#### **Pattern and persistence of anti-*Shigella* LPS IgG subclass response after**

**vaccination with *Shigella* conjugates:** Fourteen days after vaccination a single dose of the *S. sonnei* conjugate induced significant IgG1, IgG2 and IgG3 responses in 88.6%, 71.4%, and 81.8% of the vaccinees, respectively. At this time the concentrations of IgG1, IgG2 and IgG3 increased by 62.2 (from 0.9 µg/mL to 56.01 µg/mL), 25.9 (from 0.84 µg/mL to 21.8 µg/mL) and 43.5 (from 0.4 µg/mL to 17.4 µg/mL) fold respectively (figure 1). Shortly after vaccination the concentration of IgG1 was significantly higher than of IgG2, but no difference in the level of IgG1 and IgG2 was detected 6, 12 and 24 month after vaccination (Duncan's multiple range test, Alpha=0.05).

Two years after immunization the mean concentrations of IgG1 and IgG2 were still significantly higher than at the pre-vaccination step (12.14 µg/mL versus 0.9 µg/mL, and 10.2 µg/mL versus 0.84 µg/mL, respectively, Duncan's multiple range test, Alpha=0.05) (figure 1).

The pattern and relative magnitude of IgG subclass responses observed after vaccination with the *S. flexneri* conjugate were different from those found following vaccination with the *S. sonnei* conjugate. The anti-*S. flexneri* 2a IgG2 concentration was significantly higher than the concentration of both IgG1 and IgG3 throughout all the 24 months of the serological follow-up (Duncan's multiple range test, Alpha=0.05). The *S. flexneri* 2a conjugate induced a significant rise in IgG1, IgG2 and IgG3 subclasses in 81.2%, 75% and 50% of the vaccinees, respectively. At day 14 the mean concentrations of the IgG subclasses were 3.4 µg/mL for IgG1, 72.5 µg/mL for IgG2 and 1.2 µg/mL for IgG3 reaching 17, 18.2 and 15 fold increases, respectively, compared to the pre-vaccination stage (figure 2). The levels of IgG1, IgG2 and IgG3 decreased at a similar rate after the peak concentration at day 14. Two years after vaccination the mean concentrations of IgG1 and IgG2 were still significantly higher than at the pre-vaccination time (0.87 µg/mL versus 0.2 µg/mL, and 12.4 µg/mL versus 3.98 µg/mL, respectively, Duncan's multiple range test, Alpha=0.05) (figure 2).



## Discussion

Shigellosis elicits protective immunity against natural and experimental re-infection with the homologous *Shigella* serotypes (23, 8, and 2). Attempts to mimic the immune response elicited by natural infection might be an effective immunoprophylactic approach. Cohen et al (8) showed that levels of pre-existing serum antibodies (of the non-IgM class) to *Shigella* somatic antigen correlate with a reduced risk to develop shigellosis under natural conditions of exposure. We have shown that a *S. sonnei* PS-recombinant *Pseudomonas aeruginosa* exoprotein A (rEPA) conjugate vaccine, constructed to primarily induce serum IgG anti-LPS antibodies, conferred 74% protection against *S. sonnei* shigellosis (17). Purified human anti-*Shigella* antibodies were used in the present study to quantify the response to these candidate vaccines, in which covalent binding of *Shigella* polysaccharides to haptenated protein was used to strongly enhance the serum antibody response against the poorly immunogenic polysaccharide antigens (3). The *Shigella sonnei* conjugate elicited higher levels of IgG anti-LPS antibodies as compared to those induced by natural infections (about 4 fold higher).

The serum antibody response to *Shigella flexneri* 2a LPS was similar following vaccination with *S. flexneri* 2a conjugate vaccine and after homologous natural infection (9). Human IgG is composed of four structurally distinct subclasses with different biological and physiochemical properties. The distribution of IgG subclass antibodies in normal human serum reveals predominancy of IgG1 (66%) followed by IgG2 (23%), IgG3 (8%) and IgG4 (4%). IgG1 and IgG3 were found to be superior to other subclasses in complement activation, attachment to cell membranes through Fc receptors, which subsequently enhances phagocytosis, and mediation of antibody-dependent cytotoxicity (24). In a previous study we have shown the differential distribution of IgG subclasses after natural exposure to *Shigella* (9). In this study, using purified anti-*Shigella flexneri* 2a and *Shigella sonnei* antibodies, we found that the specific IgG subclass response in serum samples of volunteers receiving conjugate *S. flexneri* 2a PS-rEPA or *S. sonnei* PS-rEPA vaccine was similar in nature and character to the response elicited by natural infection. In both cases IgG2 was found to be the dominant subclass produced in response to *S. flexneri* 2a LPS/PS stimulation, whereas IgG1 and IgG2 were the main components in the response to *S. sonnei* LPS/PS produced after natural exposure, or vaccination. In a

recent study, Islam et al. (25) examined the serum IgG subclass response to *S. flexneri* serotype Y and *S. dysenteriae* serotype 1 (Shiga) 0 to 40 days after the onset of disease caused by the homologous *Shigella* serotype. The authors found a pattern of IgG subclass response to *S. flexneri* Y LPS and *S. dysenteriae* LPS in which IgG2 was the predominant IgG subclass. This pattern was similar to that detected in our study following vaccination with the *S. flexneri* 2a conjugate, and to that previously reported after natural *S. flexneri* 2a infection (9). It was different however from the IgG subclass response induced by the *S. sonnei*-rEPA or *S. sonnei* natural infection (9). The change in the relative titers of IgG1 and IgG3 from IgG2>>IgG1>IgG3>IgG4 to IgG2>>IgG3>IgG1>IgG4 during the serological follow-up, reported by Islam et al. (25) was not observed in our studies after *S. flexneri* 2a natural infection or vaccination. It must be emphasized that in both *Shigella* conjugates the carrier protein (rEPA) is similar whereas the two polysaccharides are different. Ewing and Lindberg (26) showed differences in the structure of the two polysaccharides of *S. sonnei* and *S. flexneri*. In *S. flexneri* all serotypes, except 6, are built on a common tetrasaccharide repeating unit:

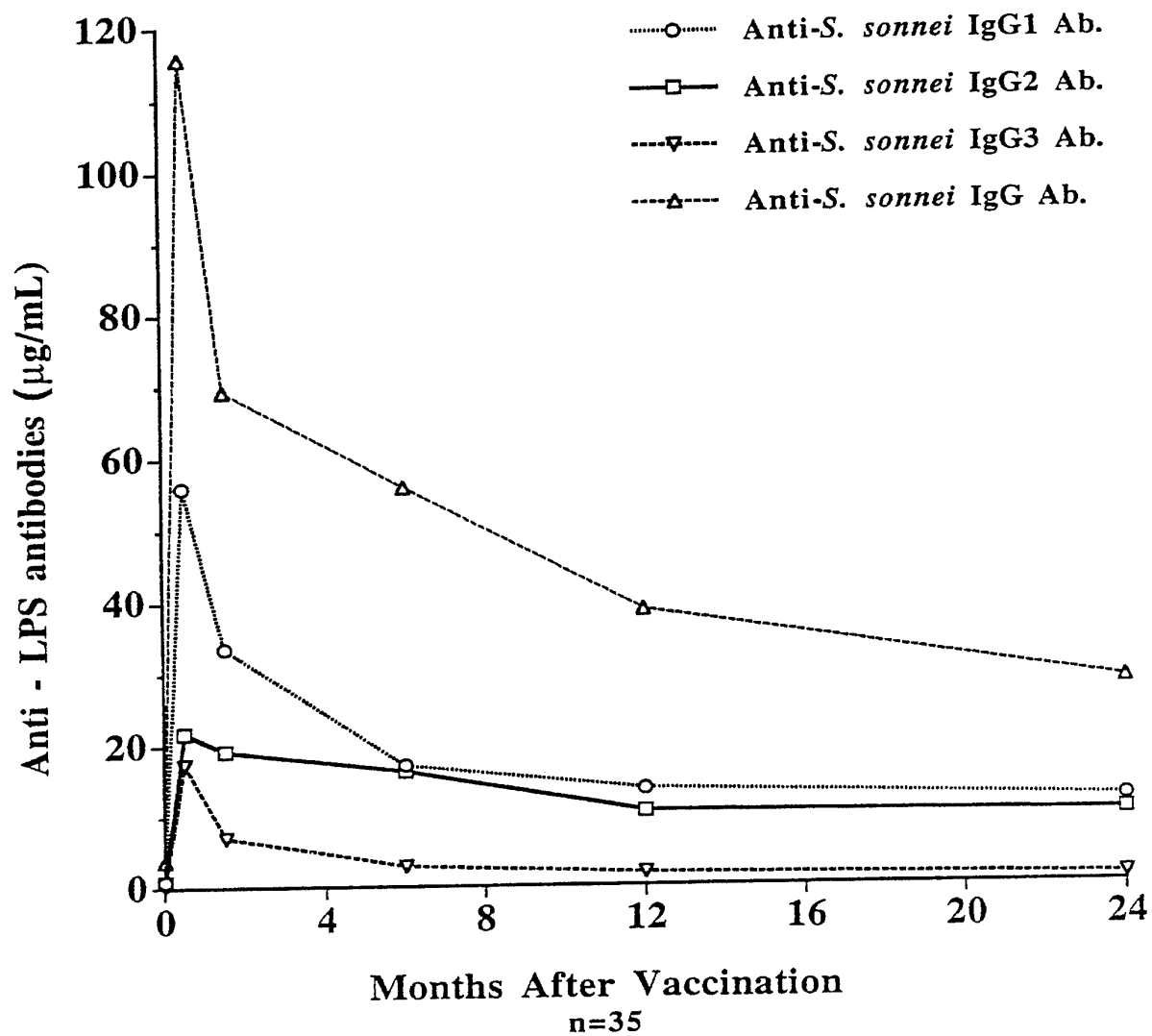
...2)- $\alpha$ -L-Rhap-(1-2)- $\alpha$ -L-Rhap-(1-3)- $\alpha$ -L-Rhap-(1-3)- $\beta$ -D-GlcpNAc-(1..., and the individual specificity of the serotypes 1a-5b is a consequence of covalent linkages of  $\alpha$ -D-glucopyranosyl and *O*-acetyl groups to different positions on the tetrasaccharide. *S. sonnei* has, in contrast, a unique disaccharide-repeating unit:

2-acetamido-2-deoxy-L-alturonic acid  $\alpha$ -(1-4)-linked to 2-acetamido-4-amino-2, 4,6-trideoxy-D-galactose that are joined by  $\beta$ -(1-3) linkages. This structure has been found so far only in *Plesiomonas shigelloides* serotype 7. Our results indicate that the origin of the differences in the IgG anti- LPS subclass response to the two species of *Shigella* (*S. sonnei* and *S. flexneri* 2a) lays in differences in the antigenic sites of the two different polysaccharides.

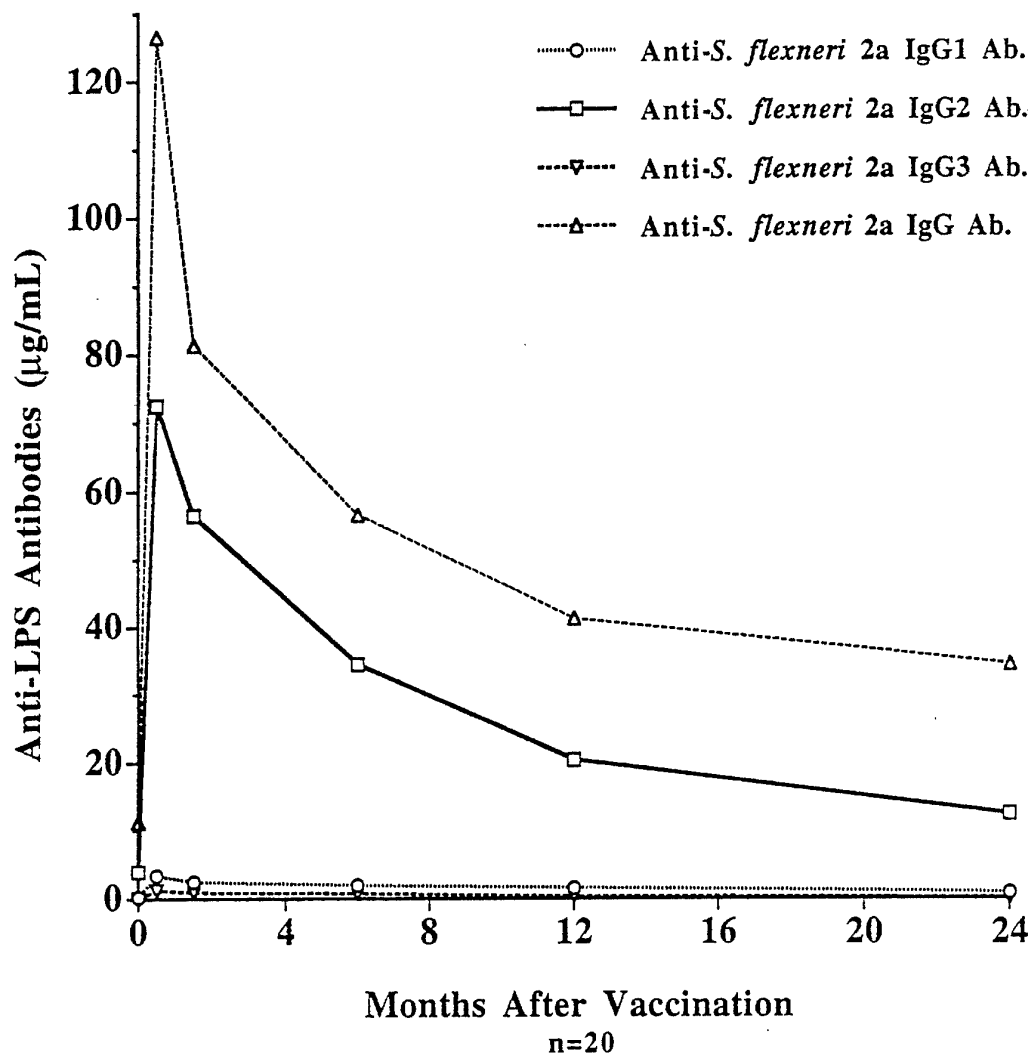
It is accepted that the magnitude and persistence of the immune response is influenced by the type and duration of exposure, severity of disease, the age of the host and the rate of antigen clearance (27). We have previously shown that the serum anti-*S. sonnei* and *S. flexneri* 2 IgG response lasts for about 6-8 months after natural *Shigella* homologous infections (7). The present study performed on the same age group, shows that the IgG and IgG specific subclasses persist for a significant longer time following vaccination with *S. sonnei* PS-rEPA and *S. flexneri* 2a PS-rEPA conjugate

vaccines. This difference could be a result of a longer immunological memory induced by parenteral delivery of *Shigella* polysaccharide conjugated to a protein (rEPA) as compared to the shorter immunological memory elicited by the LPS presentation at the mucosal site during natural *Shigella* infection.

In conclusion, we continued our study of *Shigella* conjugates and showed that the different pattern of IgG subclass response against *S. sonnei* and *S. flexneri* 2a is a result of the different structure of the two O-polysaccharides of *S. sonnei* and *S. flexneri* 2a. Further studies are required to find out if the differences in the IgG subclass response to *Shigella sonnei* and *Shigella flexneri* 2a PS's are related to a different stimulation of Th1 and Th2 and if they have any impact on the mechanism of protection exerted by the host against *S. sonnei* and *S. flexneri* 2a infections.



**Figure 1:** Dynamics of serum anti-*Shigella* IgG and IgG subclass response after vaccination with *S. sonnei* conjugate. Results are presented as geometric mean concentration of six consecutive serum samples, obtained from 35 volunteers, calculated from a slope based on eight double dilutions.



**Figure 2:** Dynamics of serum anti-*Shigella* IgG and IgG subclass response after vaccination with *S. flexneri* 2a conjugate. Results are presented as geometric mean concentration of six consecutive serum samples, obtained from 20 volunteers. calculated from a slope based on eight double dilutions.

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### Study 3

**Killing efficiency of polymorphonuclear cells against *Shigella sonnei* and *Shigella flexneri* 2a in the presence of specific *Shigella* antibodies.**

#### **Background.**

We have previously shown that in sera of naturally infected subjects and in subjects that were immunized with a conjugate *Shigella* vaccines, the patterns of IgG subclass anti-*Shigella* LPS response may vary among different *Shigella* species. In this study we aimed to examine the efficiency of phagocytes to bind and kill *Shigella* organisms, in the presence or absence of specific homologous antibodies and to examine the impact of different ratios of purified anti-*Shigella* IgG subclasses on the ability to mediate anti-*Shigella* activity by phagocytes.

#### **Methods**

**Purification of specific *Shigella* antibodies:** Specific *Shigella* antibodies were purified and quantified as described in Study 1.

**Polymorphonuclear cell activity against *Shigella* organisms mediated with human purified anti-*Shigella* antibodies:** *Shigella* organisms ( $1 \times 10^4$ ) were mixed with different dilutions of pooled anti-*Shigella* purified human antibodies and added to  $1 \times 10^6$  polymorphonuclear (PMN) cells in DMEM medium supplemented with 3.5 mg/ml BSA. The mixture was centrifuged at 350g at 4°C for 30 seconds to maximize contact between the PMN and the bacteria and allowed to incubate for 1hr at 37°C, 7.5% CO<sub>2</sub>. PMN Cells were then killed by sonication and three aliquots were plated on blood agar base to allow colony formation. The binding assay included plating samples of the supernatant after the test incubation.

## Results

Binding of *Shigella* to human polymorphonuclear cells correlated with increasing concentrations of specific anti LPS purified antibodies. Tests performed in the presence of specific antibodies and monocytes/macrophages showed strong attachment of *Shigella* to the cells, followed by cell death.

It was found that the relative serum concentration of IgG1 to homologous LPS is crucial to the capability of phagocytes to kill *Shigella*.

Bacteria	IgG 2 to IgG 1 ratio	Percent killed
<i>Shigella flexneri</i> 2a	7:1	25 %
<i>Shigella flexneri</i> 2a	2:1	61 %
<i>Shigella sonnei</i>	1:1.44	79 %

#### Study 4

##### Development of a PCR protocol for rapid detection of Shiga-like toxins I and II (SLT I and SLT II) in stool specimens.

**Background:** A PCR protocol for detection of SLT I and SLT II genes in strains of *E. coli* was developed in our laboratory and tested for specificity with a collection of enterobacteriaceae. Further on we used this protocol to examine stool samples collected from children and caretakers involved in an outbreak of *E. coli* O157-associated diarrhea in a day care center.

**Methods:** The primers used for detection of SLT I and SLT II were synthesized according to a published sequence of SLT I (Gene bank accession no. M16625) and SLT II (Gene bank accession no. XO7865) and are depicted in the following table:

	Primer	PCR product
SLT I	5' CAGTTAATGTGGTGGCGAAG	894 bp
	3' CTGCTAATAGTTCTGCGCATG	
SLT II	5' CTTCGGTATCCTATTCCGG	478 bp
	3' GGATGCATCTCTGGTCATTG	

The PCR protocol was optimized in our laboratory using *E. coli* O157 SLT II+ as a positive control. The best results were obtained using the following protocol: (a) 97°C for 30 second; (b) 29 cycles of 30 seconds at 97°C, 35 seconds at 50°C, and 1 minute at 72°C; (c) 5 minutes at 72°C. PCR products were run on 0.8% agarose gel. The specificity of the assay was tested using the following strains: *Salmonella enteritidis*, *Salmonella muenchen*, *Salmonella hadar*, *Salmonella typhimurium*, *Enterobacter*, *Proteus vulgaris*, *Proteus mirabilis*, *Klebsiella*, ETEC (CI-365 ST+, LT+), EPEC, EIEC, *Shigella sonnei*, *Shigella flexneri* 2a, *Shigella flexneri* 1b, *Shigella dysenteriae* 1.

Preparation of bacteria for the assay: Bacteria were inoculated directly into BHI tubes. The tubes were incubated for 4 hrs at 37°C and then boiled for 10 min before applied to the PCR.

Stool samples processing: Peri-anal post-defecations rectal swabs were obtained and used to inoculate BHI tubes. The tubes were incubated for 4 hrs at 37°C and then boiled for 10 min before applied to the PCR.

**Results:** Specificity of the assay: *E. coli* O157 SLT II+ showed positive reaction with SLT II primers giving the expected band at a size of 480 bp while the DNA extracted from all the other enterobacteriaceae mentioned above was not amplified by the same primers. We are currently working to evaluate the application of the SLT I set of primers on DNA extracted from SLT I positive *E. coli* O157 strains.

Use of PCR to identify *E. coli* O157 SLT II in stool samples: During the winter of 1997, an outbreak of diarrhea occurred in a daycare center at a kibbutz in northern Israel. One child in this group was hospitalized with HUS. About two weeks after the onset of the outbreak we obtained stool specimens from 8 children and 2 caretakers at this daycare center, as well as from 4 children from a nearby nursery. Specimens of two of the children from the daycare center were positive for SLT II by direct PCR. All specimens were cultured in parallel on MacConkey-Sorbitol agar for isolation of *E. coli* O157. The same stool specimens found positive for SLT II by direct PCR grew *E. coli* O157 on MacConkey-Sorbitol. These strains also produced SLT II as confirmed by a commercial kit for EHEC toxins (Meridian Diagnostics, Ohio). The two PCR positive strains were also identified as *E. coli* O157 SLT II + at the Israel National Reference Laboratory for *E. coli*.

**Conclusions:** The PCR protocol established in our lab was found to be specific for *E. coli* O157 SLT II+. This protocol allowed us to identify the presence of SLT II directly in stool samples of children involved in an outbreak of *E. coli* O157 SLT II + during the convalescent stage of disease. We are currently working to evaluate the protocol for direct detection in stool specimens of genes encoding for SLT

## Study 5

### Analysis of nalidixic acid-resistant *S. sonnei* strains.

**Background:** Between December 1996 and August 1997, seventy five nalidixic acid (NA) resistant *S. sonnei* strains were isolated from children living in the town of Kyriat Shemona and in nearby settlements of northern Israel. Most of the isolates were obtained during January and February 1997. We carried out a molecular analysis of 13 of the isolates resistant to nalidixic acid and compared them to nalidixic acid-sensitive *S. sonnei* isolates obtained in the same region during 1998.

**Methods:** Details on the subjects involved in the outbreak and on the specific isolates were obtained from the local health authority, the regional hospital where some of the subjects were hospitalized, and from the regional central laboratory. The antibiogram profiles included the standard antibiotics: Ciprofloxacin, Tetracycline, Augmentin, Nalidixic acid.

**RFLP analysis:** RFLP analysis was done according to a published method (Yavzori, M. et al. Epidemiol. Infect. 1992. 109:273-282). Briefly, chromosomal DNA extracts from the *S. sonnei* isolates were digested with either EcoR I, Bam H I, Sma I, Pvu I or Hinf I. The digested preparations were run on 0.8 % agarose gel and transferred onto GeneScreen membrane. For detection we used a P<sup>32</sup> labeled probe containing fragments of the 16s and 23s rRNA of *E. coli*.

**Plasmid profile:** Plasmids from the different strains were extracted using QIAprep Spin Plasmid Miniprep Kit (QIAGEN) and run on 0.8 % agarose gel.

**Results:** The antibiogram profile did not show any significant differences between NA resistant and sensitive strains in relation to fluoroquinolones, as all the strains were sensitive to ciprofloxacin. There were however some differences in resistance to ampicillin and tetracycline. RFLP analysis showed that all enzymes except Sma I gave identical profiles for all strains. Digestion of NA sensitive isolates with Sma I gave profiles that were different from the profiles of NA resistant strains. The plasmid profiles of all NA resistant strains contained an extra band at about 4 kb that was absent in all sensitive strains.

**Conclusions:** We dealt with the first cluster of nalidixic acid resistant-*S. sonnei* in Israel. The resistance to NA was not accompanied by significant differences in the antibiotic pattern, including the sensitivity to ciprofloxacin. However, on a molecular level, using RFLP and plasmid profile assays, there were significant differences between NA resistant and sensitive strains. We are currently working out to sequence the *gyr A* gene in resistant strains. Point mutations in this gene were previously reported in NA resistant enterobacteriaceae (J. E. Ambler, Y. J. Drabu, P. H. Blakemore and R. J. Pinney. 1993. J Antimicrob Chemother. 31:831-9, Chu, Y. W., E. T. Houang, D. J. Lyon, J. M. Ling, T. K. Ng, and A. F. Cheng. 1998. Antimicrob Agents Chemother. 42:440-3).

## Study 6

### **Immunological evaluation of the live-attenuated *S. flexneri* 2a SC602 investigational vaccine**

The Army Health Branch Research Unit has recently been involved in the immunological evaluation of the live-attenuated *S. flexneri* 2a SC602 investigational vaccine. Serum and urine samples obtained from 42 volunteers enrolled in a phase II study carried out at WRAIR and USAMRIID, have been tested at the IDF lab. A total of 288 consecutive serum and 285 urine samples obtained from vaccinees and placebo recipients were tested in a blinded fashion at the Army Health Branch Research Unit. The sera in double dilutions and duplicates were examined by ELISA for levels of *S. flexneri* 2a LPS IgG, IgA and IgM antibodies. Urine samples were assayed for titers of IgA against the same *S. flexneri* 2a LPS.

The results were transferred to Dr. Thomas L. Hale at WRAIR.